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# **Does Total Antioxidant Capacity Play a Central Role in Post-harvest Deterioration of ‘Sweetheart’ Sweet Cherry Fruit?**

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**Keywords:** ascorbic acid; antioxidant capacity; cell wall; *Prunus avium* L.; post-harvest quality; storage

## **Abstract**

‘Sweetheart’ cherries (*Prunus avium* L.) have sweet flavour, bright red colour and a distinctive heart shape which contributes to their wide commercial acceptance. They display also comparatively high firmness and long shelf-life potential. Enzyme-catalysed disassembly of cell walls has been generally targeted as the main factor accounting for ripening-related changes in fruit firmness and overall texture, but the biochemical mechanisms involved in this process appear to include additional factors such as oxidative scission of cell wall polysaccharides. In this study, ‘Sweetheart’ fruit were hand-collected at commercial maturity, and kept at 0 °C for 15 or 30 days plus 3 days at 20 °C to mimic their retail period. Firmness, weight loss and juiciness, together with the incidence of decay and stem browning, were chosen as indicators of commercial quality of fruit. Cell wall materials were extracted and fractionated, related enzyme activities were assessed, and ascorbic acid content as well as radical scavenging activity (RSA) were also determined. Fruit displaying higher RSA showed higher values for firmness and lower weight loss, decay and stem browning incidence. Higher firmness levels were associated to higher RSA and to yields of the cell wall fractions enriched in covalently-bound pectins and hemicelluloses.  $\beta$ -galactosidase, pectinmethylesterase and endo-1,4- $\beta$ -D-glucanase activities correlated inversely to firmness, while pectate lyase activity appeared to be relevant for solubilisation of cell wall materials and was inversely related to ascorbic acid content.

## **INTRODUCTION**

Commercial quality of sweet cherry (*Prunus avium* L.) fruit comprises aspects related to appearance (colour of fruit and stems, freedom from defects and infections) as well as to taste (soluble solids content, titratable acidity, aroma, flavour) (Romano et al., 2006). These fruit are particularly rich in bioactive compounds such as anthocyanins and polyphenolics (McCune et al., 2011), which represent an additional commercial value for consumers. Handling options and marketing possibilities of these fruit, though, are restricted by rapid softening rates associated to high susceptibility to infections and mechanical bruises.

Current understanding of the biochemical processes involved in post-harvest firmness changes in cherry fruit is still poor. Historically, enzyme-mediated modifications in cell wall composition and structure have been targeted as putatively main determinants of firmness and texture changes (Goulao and Oliveira, 2008), but there is experimental evidence that non-enzymic mechanisms are also involved in the scission of cell wall polysaccharides, in which ascorbic acid and its oxidation derivatives apparently play a key role (Fry, 1998). With this study, we meant to identify enzymatic and non-enzymic factors potentially related to firmness loss and post-harvest deterioration of sweet cherry fruit.

## MATERIALS AND METHODS

### Plant material and quality analyses

Cherry fruit (*Prunus avium* L. cv. 'Sweetheart') were sampled in 2009 at commercial harvest time (June 25<sup>th</sup>) from an orchard located in Corbins, in the area of Lleida (NE Spain). Uniform and defect-free fruit were stored at 0 °C and 92% relative humidity under regular air for 15 or 30 days, after which samples were placed at 20 °C to simulate shelf life. In order to induce quality differences, a batch of fruit was packed within low-density (25 µm) microperforated polyethylene (PE) bags. Two replicate samples (2 kg) were used per each combination of factors (cold storage period × shelf life period). Analyses were carried out at harvest, as well as 0 (henceforth, 15+0 and 30+0 fruit) and 3 (henceforth, 15+3 and 30+3 fruit) days after removal from storage. For biochemical analyses, 30 fruit per treatment were stoned at each sampling date, frozen in liquid nitrogen, freeze-dried, powdered, and kept at -80 °C until processing.

Average fruit weight at harvest was 9.33 g. Weight of 30 cherries was determined jointly at harvest and at each analysis date in order to determine weight loss (%) regarding harvest date. Firmness was measured with a Durofel DFT 100 durometer (Agro-Technologie, Forges Les Eaux, France) fitted with a 5.64-mm tip, on two opposite faces on the cheek region of 30 fruit, and results were expressed as Durofel units (1-100) (Table 1). For the assessment of juiciness, three replicate samples (10 fruit each) per treatment were stoned and squeezed until no more juice was released. After filtration, the volume of juice recovered was measured, and expressed as mL 100 g<sup>-1</sup> fresh weight. The incidence of fungal decay was expressed as percentage of fruit affected.

### Cell wall analyses

The phenol:acetic acid:water (2:1:1, w/v/v) (PAW) method (Redgwell et al., 1992) was used for the extraction of cell wall materials (CWM) from lyophilised tissue (3 g). The PAW-insoluble pellet was washed, filtrated, lyophilised and weighed to determine yield of insoluble cell wall materials (CWM). The PAW-soluble fraction (PAW<sub>sf</sub>) was recovered, lyophilised and weighed. Yields were expressed as % (w/w) FW. Insoluble CWM (100 mg) were further fractionated by sequential extraction with water, 0.05 M cyclohexane-trans-1,2-diamine tetra-acetate (CDTA), 0.05 M Na<sub>2</sub>CO<sub>3</sub>, and 4 M KOH (Selvendran and O'Neill, 1987), in order to recover different cell wall polymer fractions, and expressed as % (w/w) CWM. Samples (30-35 mg) of pectin-containing fractions were hydrolysed and analysed for uronic acid and neutral sugar content. For the extraction of polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15), pectinmethylesterase (PME; EC 3.1.1.11), pectate lyase (PL; EC 4.2.2.2), β-galactosidase (β-Gal; EC 3.2.1.23), α-L-arabinofuranosidase (AFase; EC 3.2.1.55), β-xylosidase (β-Xyl; EC 3.2.1.37) and endo-1,4-β-D-glucanase (EGase; EC 3.2.1.4) activities, a 10% (w/v) homogenate was prepared from lyophilised tissue (100 mg). All extraction and assay procedures were as described previously (Ortiz et al., 2011). Results were given in terms of specific activity (activity units mg<sup>-1</sup> protein).

### Antioxidant status analyses

Total antioxidant capacity was measured from dry tissue (200 mg) in terms of radical scavenging activity (RSA) by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as described in Oms-Oliu et al. (2009). The colorimetric ascorbate assay (Gillespie and

Ainsworth, 2007) was used to analyse the contents of ascorbic (AA) and dehydroascorbic (DHA) acid from lyophilised tissue (40 mg), and data were given as  $\mu\text{mol gDW}^{-1}$ . Total phenolics were analysed from samples (250 mg) of dry tissue as described in Luthria et al. (2006), and results expressed as  $\text{mg g}^{-1}$  DW. Tissue samples (50 mg) were also homogenised in 10 mL methanol-HCl-water (50:1:49, v/v/v), incubated in the dark at 4 °C, and centrifuged prior to spectrophotometric estimation of total anthocyanin content ( $\text{mg g}^{-1}$  DW). Acetaldehyde content was assessed in fruit juice (5 mL) according to Ke et al. (1994), and expressed as  $\mu\text{L L}^{-1}$ .

### **Statistical analysis of data**

A multifactorial design with treatment, storage and shelf life periods as factors was used to test data by analysis of variance (GLM-ANOVA procedure) with the Statistical Analysis System (SAS version 9.1, SAS Institute, Inc., Cary, NC, USA), and means were separated by the Fisher's LSD test at  $P \leq 0.05$ . Principal component analysis (PCA) was used to provide a general overview of the information contained in the dataset obtained. Partial least squares regression (PLSR) was used as a predictive method to relate a matrix of dependent variables ( $Y$ ) to a set of explanatory variables ( $X$ ). Unscrambler version 7.6 software (CAMO ASA, Trondheim, Norway) was used for developing these models.

## **RESULTS AND DISCUSSION**

### **Fruit quality and antioxidant properties after cold storage**

Cherries were stored for up to one month in order to cause differences in firmness and other storability indicators, with the aim of facilitating the identification of biochemical factors involved in the softening and deterioration processes.  $\text{O}_2$  and  $\text{CO}_2$  concentrations within the PE packages were modified to 20.32% and 0.32%, respectively, after 30 days at 0 °C. Firmness at harvest was over 80 Durofel units (Table 1) and declined thereafter significantly, though not sharply, in stored fruit, higher levels being generally preserved in PE-packed samples. Firmness increased significantly in fruit submitted to cold storage for 30 days as compared with those stored for 15 days. Similarly, the highest juiciness levels were found for 30+0 samples. Although fruit juiciness is generally regarded as desirable, it may be also indicative of over-ripening and tissue disintegration. Actually, 30+0 samples contained higher levels of acetaldehyde (Table 2), which is known to promote fruit softening in some cases (reviewed in Pesis, 2005), and also scored higher for off-flavours (data not shown).

Total antioxidant activity, measured as RSA, decreased significantly after harvest, although no differences were found between fruit stored for 15 and 30 at 0 °C. RSA declined further during the subsequent post-storage period at 20 °C (Table 2), and PE-packaged samples retained higher levels in all cases. Contrarily, levels of ascorbic acid, both reduced (AA) and oxidized (DHA), were significantly affected by storage period as well as by sample packaging. Levels of AA declined noticeably after 30 days at 0 °C, concomitantly with strong increases in DHA (Table 2), the oxidized form of ascorbate, while packaging largely prevented these changes. The content of total phenolics and anthocyanins increased during the first 15 days of cold storage as compared with levels at harvest, but declined again in fruit stored for 30 days, although PE-packaged cherries retained higher contents of both compound types (Table 2). The correlations between total antioxidant activity and either AA, phenolics or anthocyanin contents were not

particularly significant, contrarily to previous observations for developing cherry fruit (Serrano et al., 2005).

When quality attributes and antioxidant properties were included in a PCA model, the corresponding correlation loadings plot (Fig. 1) revealed that higher firmness was associated to higher RSA and, to a lesser extent, AA. RSA correlated inversely to decay, stem browning and weight loss, suggesting that antioxidant properties may be important for delaying firmness loss and associated alterations. Therefore we extracted, fractionated and analysed cell wall materials in order to assess the possible relationship between cell wall metabolism and antioxidant properties of fruit.

### **Cell wall metabolism after cold storage**

Cell wall fraction yields, together with RSA, AA, DHA and acetaldehyde contents, were used as the potentially explanatory variables (*X*-matrix) to develop a regression model of firmness and associated attributes. The corresponding correlations loadings plot (Fig. 2) shows that the model explained up to 86% of total variability in the *Y*-matrix, with principal component 1 (PC1) accounting alone for 79% thereof. Firmness was associated to RSA and to the yields of Na<sub>2</sub>CO<sub>3</sub>- and KOH-soluble fractions (NaCO<sub>sf</sub> and KOH<sub>sf</sub>, enriched respectively in covalently-linked pectins and in matrix glycans) and, to a lower extent, to AA contents and CDTA<sub>sf</sub> yields. In turn, decay and weight loss were inversely correlated to RSA and to NaCO<sub>sf</sub> and KOH<sub>sf</sub> yields, which also suggests a role for oxidative status in the progress of fungal infections. Interestingly, a higher degree of cell wall solubilisation, as indicated by yields of PAW<sub>sf</sub>, was associated to higher contents of oxidised ascorbate (DHA) and acetaldehyde, consistent with previous reports on the relationship between fruit softening and these metabolites (Fry, 1998; Pesis, 2005).

Ripening-related modifications in cell wall composition and structure are attributed usually to the coordinated action of a number of specific enzymatic and non-enzymatic proteins on different cell wall polysaccharides which eventually lead to cell wall disassembly. The controlling mechanisms involved in this event, though, appear to vary widely among species (Goulao and Oliveira, 2008) and to include additional factors. Hydroxyl radicals, for instance, have been shown to contribute to the oxidative, non-enzymatic scission of cell wall polysaccharides (Fry, 1998). Since data indicated that firmness and other storage-related attributes were associated to the yield of specific cell wall fractions (Fig. 2), an additional regression model was developed for yield and composition of cell wall fractions isolated from fruit, in which seven different pectolytic and non-pectolytic enzyme activities, together with RSA and AA, DHA and acetaldehyde contents, were used as the potentially explanatory variables (*X*-matrix). This model revealed some interesting correlations (Fig. 3).

### **A cross-talk between enzymic and non-enzymic factors in the modulation of cell wall changes after cold storage?**

The yields of the Na<sub>2</sub>CO<sub>3</sub>-soluble fraction, associated to fruit firmness (Fig. 2), were correlated inversely to  $\beta$ -Gal activity levels, suggesting a role in firmness loss. It has been suggested that the highly branched structure of pectins may restrict the access of pectin backbone-acting enzymes to their substrates, thus limiting pectin degradation and solubilisation in spite of high levels of pectolytic activity (Goulao and Oliveira, 2008). Galactosyl- and arabinosyl-containing side-chains are thought to control pore size in the cell wall and to protect cell wall polysaccharides from extensive depolymerisation by pectolytic enzymes. Indeed,  $\beta$ -Gal acts on galactosyl-rich side-chains in pectins (Gerardi

et al., 2012), its reported pH optimum of 4.0 being consistent with the idea of increased activity upon ripening-associated decrease of apoplastic pH. The observation of an association between the activities of  $\beta$ -Gal and of the pectin backbone-acting enzyme PME (Fig. 3) supports the view that enzymes acting on pectin side-chains facilitate pectin solubilisation by modulating cell wall porosity. In turn, PME action itself contributes to changes in pH and electric charge of the apoplast, thus modifying substantially the activity and mobility of other cell wall proteins.

PME activity removes methyl groups held by a large number of the uronic acid residues presents in pectins, this demethylation being required for subsequent PG- and PL-catalysed cleavage of galacturonic acid residues from the backbone of pectic polymers. The observation that PME activity was inversely correlated to the firmness-determining  $\text{NaCO}_{\text{sf}}$  is consistent with a key role in firmness loss. The pectin backbone-acting PL activity (and, to a lesser extent, PG) was associated to pectin solubilisation as indicated by the yields of the cell wall fractions soluble in PAW and water (Fig. 3), as well as to uronic acid content in the  $\text{CDTA}_{\text{sf}}$ . The latter observation has been also reported for other stone fruit species such as peach and nectarine (reviewed in Goulao and Oliveira, 2008), and suggested to arise from remobilisation and transient reallocation of insoluble cell wall materials from the  $\text{NaCO}_{\text{sf}}$  to the  $\text{CDTA}_{\text{sf}}$  during softening.

In spite of being associated to solubilisation of cell wall materials, PL and PG correlation with  $\text{NaCO}_{\text{sf}}$  yields, and thus with firmness, was poor, showing that solubilisation of cell wall polymers may be related to tissue softening through an indirect mechanism. These two enzyme activities, together with PME and the hemicellulose-degrading EGase, were inversely correlated with the contents of ascorbic acid, again suggesting that antioxidant activity may have a role in preventing extensive cell wall disassembly. Ascorbate-generated hydroxyl radicals can cause non-enzymic scission of cell wall polysaccharides and thus contribute to fruit softening (Fry, 1998), and de-esterified pectin is reportedly more susceptible to ascorbate-induced scission *in vitro* than methylesterified pectin. This is also supported by the observation that higher PL, PME and EGase activity levels were associated to higher contents of dehydroascorbic acid.

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## Tables

Table 1. Some quality attributes of 'Sweetheart' cherries at harvest and after cold storage.

			Days (0 °C + 20 °C)			
			15+0	15+3	30+0	30+3
Firmness (Durofel units <sup>a</sup> )	81.2	Control	74.6 c	73.2 c	76.9 b	76.4 b
		PE	77.6 b	75.9 b	79.0 a	76.5 b
Weight loss (%)	-	Control	1.0 e	6.9 a	1.8 d	5.1 b
		PE	0.6 e	4.3 c	0.5 e	4.6 c
Juiciness (mL 100g <sup>-1</sup> FW)	61.3	Control	61.9 c	63.2 b	65.7 a	56.3 e
		PE	59.8 d	57.7 e	65.1 a	58.9 d
Decayed fruit (%)	-	Control	0.0 e	26.7 c	23.3 c	60.0 a
		PE	4.0 d	40.0 b	10.0 d	40.0 b

<sup>a</sup> (Durofel units: 1, no resistance - 100, maximum resistance).

Values represent means of 30 (firmness) or 3 (juiciness) replicates. For weight loss assessment, the same 30 fruit were weighed jointly at harvest, upon removal from cold storage and 3 days thereafter. The incidence of decay and stem browning were evaluated on the total number of fruit. Mean values for a given parameter followed by different letters are significantly different at  $P \leq 0.05$  (LSD test).

Table 2. Radical scavenging activity and content of antioxidant capacity-related compounds in ‘Sweetheart’ cherries at harvest and after cold storage.

	Harvest	Treatment	Days (0 °C + 20 °C)			
			15+0	15+3	30+0	30+3
RSA <sup>a</sup> (%)	37.3	Control	27.8 b	23.7 c	27.1 b	23.2 c
		PE	34.5 a	31.6 ab	35.0 a	29.9 b
AA <sup>b</sup> (μmol g <sup>-1</sup> DW)	2.01	Control	2.17 a	1.73 b	0.93 d	1.00 d
		PE	1.60 b	1.25 c	1.52 b	1.54 b
DHA <sup>b</sup> (μmol g <sup>-1</sup> DW)	0.0	Control	0.07 e	0.00 f	1.26 a	0.57 b
		PE	0.22 c	0.16 d	0.12 d	0.21 c
Phenolics (mg g <sup>-1</sup> DW)	9.72	Control	11.62 a	10.48 b	10.39 b	9.33 c
		PE	10.79 b	11.10 ab	11.50 a	10.57 b
Anthocyanins (mg g <sup>-1</sup> DW)	3.96	Control	5.27 a	5.19 a	3.52 c	4.12 b
		PE	5.22 a	4.99 a	4.92 a	4.87 a
Acetaldehyde (μL L <sup>-1</sup> )	1.52	Control	1.53 c	1.43 c	2.10 a	1.67 b
		PE	1.70 b	1.04 d	1.44 c	1.46 c

<sup>a</sup> RSA: radical scavenging activity.

<sup>b</sup> AA: reduced ascorbic acid; DHA: oxidised ascorbic acid (dehydroascorbic acid).

Values represent means of 3 replicates. Mean values for a given parameter followed by different letters are significantly different at  $P \leq 0.05$  (LSD test).

## Figures

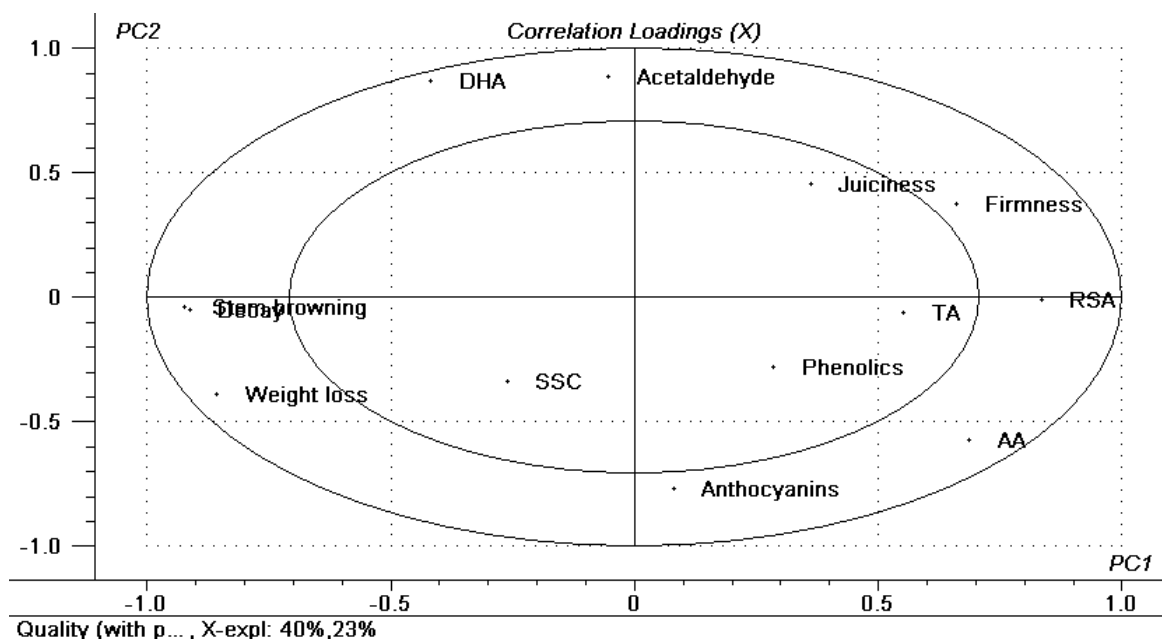


Fig. 1. Correlation loadings plot of PC1 vs. PC2 corresponding to a PCA model for commercial quality attributes and antioxidant capacity in ‘Sweetheart’ sweet cherry fruit at harvest and after cold storage (AA, ascorbic acid; DHA, dehydroascorbic acid; RSA, radical scavenging activity; SSC, soluble solids content; TA, titratable acidity).



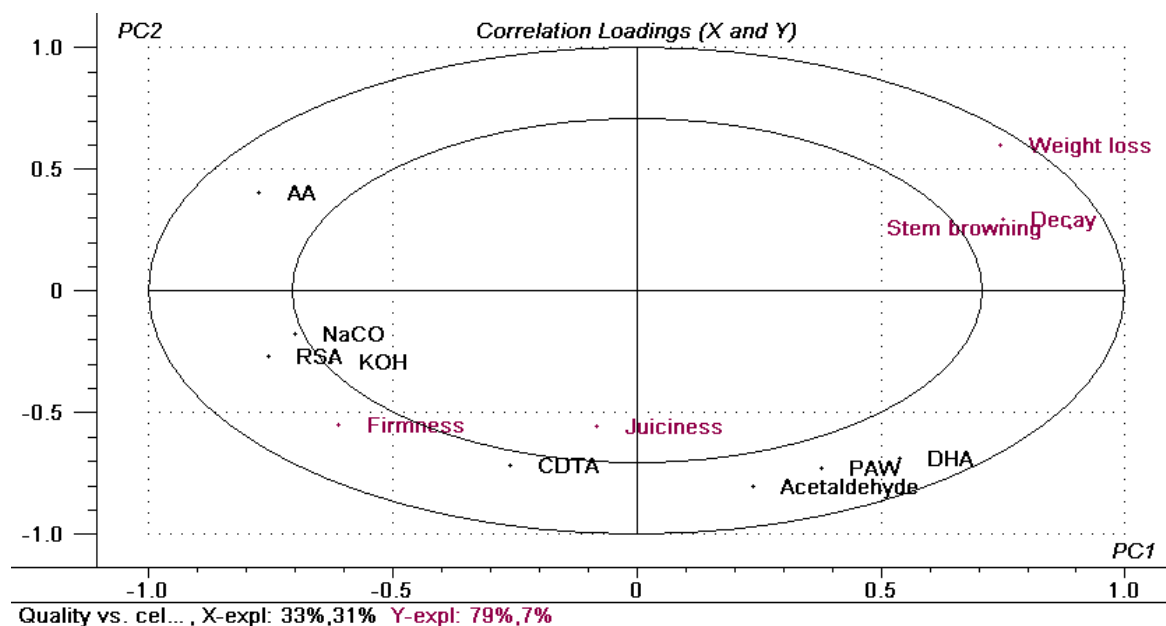


Fig. 2. Correlation loadings plot of PC1 vs. PC2 corresponding to a PLSR model for storage potential-related attributes (Y variables) vs. cell wall fractions and antioxidant properties (X variables) in 'Sweetheart' sweet cherry fruit at harvest and after cold storage (AA, ascorbic acid; DHA, dehydroascorbic acid; RSA, radical scavenging activity; PAW, PAW-soluble cell wall material; CDTA, chelator-soluble fraction; NaCO, Na<sub>2</sub>CO<sub>3</sub>-soluble fraction; KOH, KOH-soluble fraction).

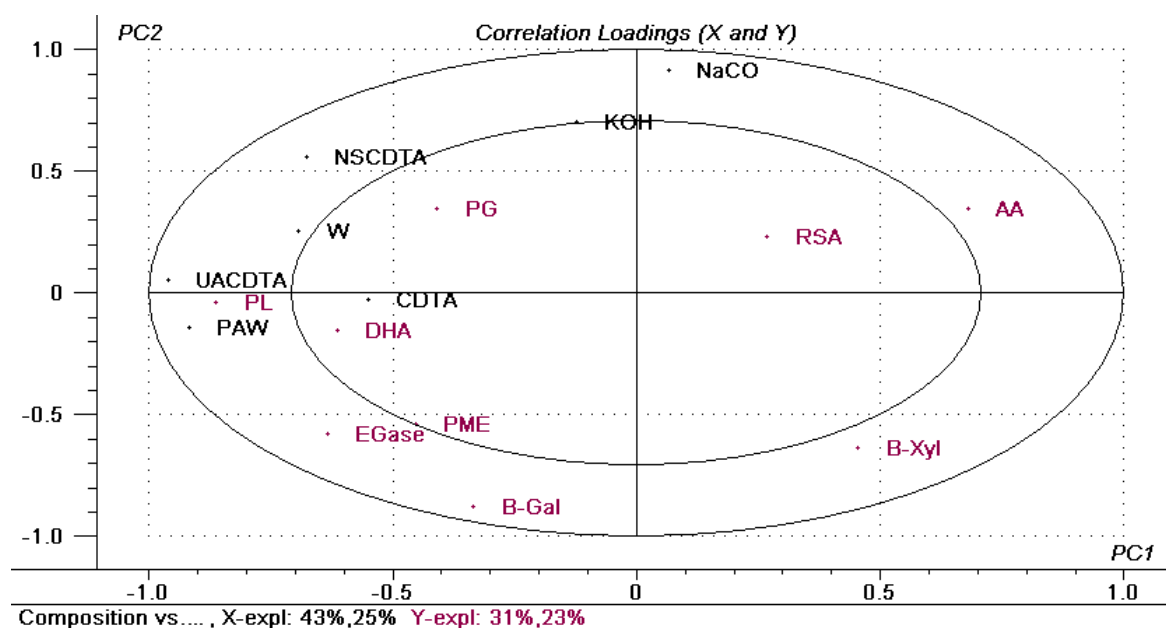


Fig. 3. Correlation loadings plot of PC1 vs. PC2 corresponding to a PLSR model for cell wall composition (Y variables) vs. cell wall-modifying enzyme activities and antioxidant properties (X variables) in 'Sweetheart' sweet cherry fruit at harvest and after storage (AA, ascorbic acid; DHA, dehydroascorbic acid; RSA, radical scavenging activity; PAW, PAW-soluble cell wall material; W, water-soluble fraction; CDTA, chelator-soluble fraction; NaCO, Na<sub>2</sub>CO<sub>3</sub>-soluble fraction; KOH, KOH-soluble fraction; UACDTA and NSCDA, uronic acid and neutral sugar contents in the CDTA-soluble fraction).